The Analyst
FULL PAPER

Comparison of biota sample pretreatments for arsenic speciation with coupled HPLC-HG-ICP-MS

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Received 8th November 1999, Accepted 4th January 2000
Published on the Web 9th February 2000

Soxhlet extraction has been compared to sonication and microwave assisted extraction of arsenite, arsenate, dimethylarsenic, monomethylarsenic and arsenobetaine from biota samples (oyster, cockle, mussel and fresh water alga) and Certified Reference Materials TORT-1 and CRM 627, using methanol and methanol–water mixtures. A clean-up procedure using hexane partitioning was compared to the use of 0.45 μm filter membranes, ultrafiltration (10 kDa) and C-18 cartridges. Measurements were carried out using coupled HPLC-(UV)-HG-ICP-MS. The best results were obtained for Soxhlet extraction (150 ml of a (1 : 1) methanol–water mixture for 16 h) compared to both sonication (20 ml of methanol or methanol–water mixtures (1 : 3), (1 : 1) and (3 : 1) during 20 min) and microwave (20 ml of methanol or methanol–water mixtures (1 : 3), (1 : 1) and (3 : 1) during 10 min at 150 W). Hexane partitioning was found to be superior to the other clean-up procedures. Recoveries higher than 71% were obtained depending on the type of sample. An unknown compound was found to be the main arsenic species in the marine samples.

1 Introduction

Arsenic in biota samples is mainly found in the form of organic species, as has been summarized by Cullen and Reimer.[1] Arsenobetaine (AsB) was firstly isolated and identified in the Western rock lobster by Edmonds *et al.*[2] This non toxic compound represents the main species of arsenic in a number of marine organisms such as fish, molluscs and crustaceans.[3,4] It is assumed that the presence of arsenobetaine in these organisms is the result of a biological cycle in which arsenate already present in the seawater is methylated to monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA), then converted into arsenic-containing ribofuranosides (arsenosugars) by seaweed and further transformed into arsenobetaine within the food chain.[5] The marine cycle of arsenic continues with the microbiological degradation of AsB, first to methylated species and finally to inorganic arsenic.[6] In some organisms, such as seaweed and shellfish (oysters), a higher content of arsenosugars than that of AsB is frequently found.[7,8] Methylated compounds, DMA and MMA, are present in a minor percentage, as well as the inorganic species arsenite As^{III} and arsenate As^{V}. For terrestrial organisms, the arsenic cycle seems to finish mainly in the formation of methylated species (MMA and DMA), although AsB has also been identified in some types of mushrooms.[9]

The analytical approaches for the speciation of arsenic in biota samples generally involve the use of separation techniques coupled with a sensitive atomic detector. High performance liquid chromatography (HPLC) has been successfully directly coupled to inductively coupled plasma-optical emission spectrometry (ICP-OES)[10] and inductively coupled plasma-mass spectrometry (ICP-MS).[11] Other atomic detectors, atomic absorption spectrometry (AAS)[12] and atomic fluorescence spectrometry (AFS)[13] include hydride generation (HG) as an intermediate step, thus converting the arsenic compounds into volatile arsines prior to the detection. However, arsenobetaine and arsenosugars do not form volatile hydrides and the destruction of the organic part of the molecules before hydride generation is required. This has been achieved by the use of both on-line microwave digestion[14] and photooxidation with UV radiation.[15,16] Other approaches based on hydride generation of the arsines and their preconcentration using cold trapping (CT) provide very good sensitivity for inorganic arsenic, DMA and MMA,[17] but do not allow the determination of arsenobetaine and arsenosugars and therefore are not usually considered for biota analysis.

The sample extraction and clean-up procedures constitute a crucial step when biota samples are considered, due to possible analyte losses, changes of the species or incomplete extraction of the arsenic compounds, which may lead to poor or erroneous results. The literature describes the use of enzymatic digestion[3,18] or methanol, methanol–water, methanol–water–chloroform mixtures, either with manual agitation, vortex agitation, or sonication,[19–21] the methanol–water (1 : 1) mixture in an ultrasonic bath being the most widespread in use. Afterwards, the supernatant is separated from the residue by centrifugation.[22] Moreover, sample clean-up is necessary to remove the lipid content of tissues, in order to maintain the performance of the chromatographic separation. This has been carried out using solid phase extraction with C-18 cartridges,[8] Florisil and solvent partitioning with diethyl ether and petroleum ether,[20] filtration[23] and ultrafiltration.[24]

The aim of this work is to compare different extraction and clean-up procedures to achieve the speciation of arsenic (arsenite, arsenate, arsenobetaine, monomethylarsenic and dimethylarsenic) in fresh water algae (*Zyngogomium* sp.) and seawater bivalves (mussels, *Mytilus edulis*, oysters, *Crassostrea gigas* and cockles, *Cerastoderma edulis).* Soxhlet, a general extraction technique for normalized methods dealing with solid samples, was considered in this work as a possible sample pretreatment for arsenic speciation. The capacity of Soxhlet extraction was tested against other more widely used procedures, such as sonication and microwave assisted extraction. Hexane partitioning was considered for sample clean-up altogether with more tedious and expensive procedures described in the literature, such as C-18 cartridges, filtration and ultrafiltration. Samples were collected along the Southwest coast of Spain (Atlantic coast and Odiel River). Coupled HPLC-HG-ICP-MS was employed for the analysis, optimizing the different instrumental variables involved. Optionally, on-line photooxidation after chromatographic separation was employed

for analysis of arsenobetaine and also allowing the detection of other organic arsenic species.

2 Experimental

2.1 Apparatus

The HPLC system consisted of a quaternary HPLC pump (Varian, San Fernando, CA USA) equipped with a 250 mm × 4.1 mm strong anion exchange column (Hamilton, Reno, NV, USA). Sample introduction was performed using a Rheodyne 7125 injection valve fitted with a 200 μl loop (Rheodyne, Cotati, CA, USA). Hydride generation was carried out adding an acid and a reductant solution by means of a peristaltic pump (Minipuls 3, Gilson, Villiers, Le Bel, France). The formed arsines were separated using a glass gas–liquid separator (PS Analytical, Kent, UK). Atomization of the arsines occurred in the argon plasma torch of a ICP-MS 4500 (Hewlett-Packard, Palo Alto, CA, USA). Photooxidation was achieved using a 8 m long Teflon tube wrapped around a low pressure Hg lamp that emits UV radiation at 254 nm (TNN 15 W, Heraeus, South Plainfield, NJ, USA). Water was obtained from a Milli-Q Gradient purification system (Millipore, Bedford, MA, USA). Filter membranes of 0.45 μm (Lida, Kenosha, WI, USA) and centrifuge filters 10 000 molecular weight cut-off (MWCO) (Supelco, Bellefonte, PA, USA) were tested for sample clean up.

2.2 Reagents

Stock standard solutions (1000 mg l^{-1}, as As) were prepared from arsenic trioxide (Panreac, Barcelona, Spain), sodium arsenate (Merck, Darmstadt, Germany), sodium methylarsonate (Carlo Eba, Milan, Germany) and dimethylarsinic acid (Sigma, St. Louis, MO, USA). A 10 mg l^{-1} arsenobetaine [$(CH_3)_3AsCH_2COOH$] solution was kindly provided by Dr. Foulkes (University of Plymouth, UK). HPLC mobile phase was prepared from KH_2PO_4 and $K_2HPO_4{\cdot}3H_2O$ (Merck). $NaBH_4$ (Panreac) solution stabilized in NaOH (Merck) was prepared daily. $K_2S_2O_8$ was purchased from Aldrich (Milwaukee, WI, USA). HCl, HNO_3 and $HClO_4$ were of analytical grade (Merck). Hexane (Romil, Waterbeach, Cambridge, UK) was used for sample clean-up. Certified Reference Materials TORT-1 (Lobster Hepatopancreas) (National Research Council, Canada) and CRM 627 (tuna tissue) (BCR, Institute for Reference Materials and Measurements, Geel, Belgium) were employed for comparison.

2.3 HPLC-(UV)-HG-ICP-MS coupling

The separation of the arsenic standards took place in a strong anion exchange column using a 25 mM phosphate (pH 5.8) mobile phase, pumped at a flow rate of 1.1 ml min^{-1}. The elution order was As^{III}, DMA, MMA and As^{V}. Hydride generation was achieved by pumping 1% m/v $NaBH_4$ (stabilized in 1% m/v NaOH) and 1 M HNO_3 solutions, both at a flow rate of 1.0 ml min^{-1}. An auxiliary argon flow of 0.1 l min^{-1} was added after hydride generation, in order to carry the arsines to the gas–liquid separator. A second argon flow of 1.0 l min^{-1} introduced in the gas–liquid separator carried the arsines directly to the plasma torch of the ICP. The nebulization chamber was removed. The cooling argon flow was 16 l min^{-1}. Detection was performed at *m*/*z* 75. The signal output was integrated every 0.5 s. Peak areas were considered throughout the experiments.

For the detection of arsenobetaine, an on-line photooxidation step was included before hydride generation, consisting of the irradiation of UV light (λ = 254 nm) and the addition of a strong oxidant, 2% m/v $K_2S_2O_8$ in 2% NaOH at a flow rate of 0.3 ml min^{-1}. Under these conditions, the HNO_3 concentration was increased up to 4 M. A diagram of the instrumental coupling is depicted in Fig. 1. As AsB coeluted with As^{III}, its quantification was performed by doing two experiments, with and without photooxidation, quantifying As^{III} and the sum of As^{III} and AsB, respectively. AsB quantification is then done by difference.

2.4 Extraction and clean-up procedures

Several extraction procedures were tested based on the use of methanol and methanol–water mixtures. After extraction, the extractant was separated from the sample by centrifugation at 10 000 rpm during 10 min. The supernatant was decanted with a Pasteur pipette and the methanol removed using either a N_2 stream or a rotatory evaporator. Samples were diluted with distilled water to a final weight of 20 g. The clean-up procedures tested included: (i) filtration using 0.45 μm filter membranes; (ii) ultrafiltration using 10 000 molecular weight cut-off (MWCO) filters; (iii) hexane partitioning; and (iv) solid phase extraction with C-18 cartridges.

2.5 Total arsenic determination

A sample of 0.5000 g was placed in a 100 ml Kjeldahl flask, and predigested with 20 ml of concentrated HNO_3 for 4 h. Afterwards, it was heated up to 120 °C for 1 h in a sand bath. Five millilitres of $HClO_4$ were then added and the temperature raised until the appearance of white fumes (210 °C). The sample

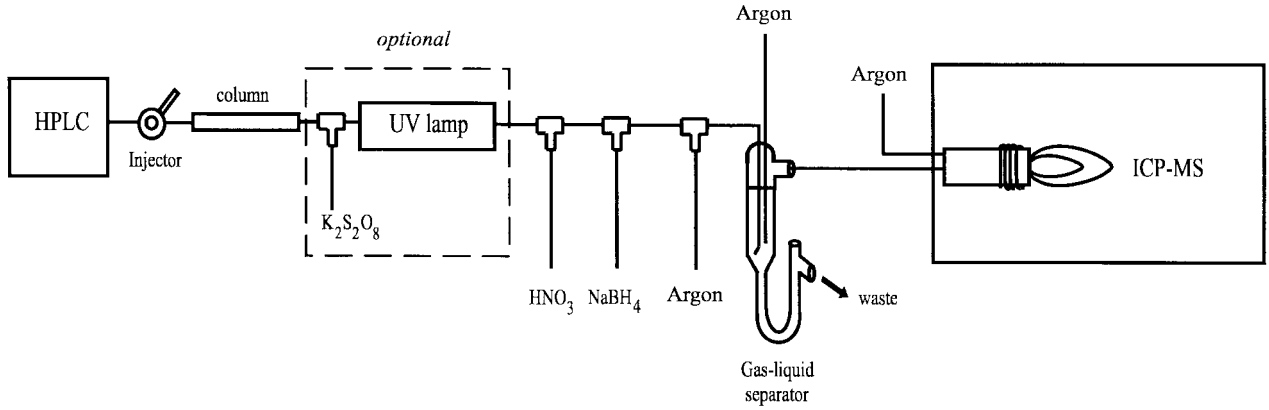

Fig. 1 Instrumental scheme of coupled HPLC-(UV)-HG-ICP-MS.

was then evaporated to dryness, rinsed with water and the solution transferred to a 50 ml volumetric flask.

2.6 Statistical treatment

The data were analyzed statistically for differences using factorial analysis of variance (ANOVA). Prior to analysis, all the data were tested for homogeneity of variance using the Barlett and Levene tests.[25] Parametric statistical test (Student's *t*-test) was applied to different hypotheses. An α-value of 0.05 was adopted as the critical level for all statistical testing giving a 95% confidence level (CSS: STATISTICA™).

3 Results and discussion

3.1 Optimization of the HPLC-(UV)-HG-ICP-MS coupling

HNO_3 and HCl solutions at concentrations ranging from 0.25 M to 5 M were tested for the hydride generation step. HNO_3 concentration did not have any significant influence on As^{III} signal (ANOVA, $P = 0.5$). However, higher sensitivities for As^V and MMA , and lower for DMA were observed when the acid concentration was increased to 1 M (*t*-test, $P < 0.01$). Similar results were obtained using HCl. This influence of the acid concentration on the signal using AFS detection has been previously described.[15] Considering that both DMA and As^V presented rather small signals and that they were strongly affected in opposite ways by the acid concentration, a compromise value of 1 M acid concentration was selected. At this concentration no difference was observed in the background signal using either HNO_3 or HCl. Hydride generation is advisable when working with samples which may contain salts, especially the Cl^- ion, which results in the ArCl interference, as has been described when coupling HPLC directly to ICP-MS.[26] The possible interference based on the formation of $ArCl^+$ was not noticeable. This fact has also been indicated by Magnuson *et al.*,[27] using 10% (m/m) HCl for hydride generation, concluding that most of the background signal was due to ^{75}As impurity.

When the photooxidation device was included to determine AsB, the acid concentration had to be increased in order to compensate for the basicity of the $K_2S_2O_8$ solution. In this case, HNO_3 and HCl concentrations were increased to an optimum value of 4 M, HNO_3 being selected as the noise of the signal background was about five times lower than that using HCl. The photooxidation step resulted also in an increase in the retention for all the arsenic compounds of *ca*. 1.5 min, as a result of the 8 m long Teflon tubing around the UV lamp. (This is noticeable comparing the chromatogams depicted in Fig. 5a and Fig. 5b.)

Concentrations of $NaBH_4$ ranging from 0.5 to 2% (m/v) were tested. An increase of the sensitivity for all of the species was obtained using a concentration of 1% (*t*-test, $P < 0.01$), which was not improved at higher concentrations (ANOVA, $P > 0.7$) and therefore was selected as the optimal value.

The auxiliary argon flow added after the hydride generation provided a fast transport of the arsines to the gas–liquid separator. As a consequence, it smoothed and improved the shape of the recorded signal. Several flows ranging from 0 to 200 ml min^{-1} were tested, a maximum peak area being obtained at a flow rate of 100 ml min^{-1} (*t*-test, $P < 0.01$). A chromatogram for AsB, DMA, MMA and As^V corresponding to the HPLC-(UV)-HG-ICP-MS coupling is depicted in Fig. 2.

Under these optimized conditions, a linear range between 1 and 750 $\mu g\ l^{-1}$ was obtained for As^V, and between 1 and 300 $\mu g\ l^{-1}$ for As^{III}, DMA and MMA. When the photooxidation step was included, linear ranges for all the species were between 1 and 750 $\mu g\ l^{-1}$, due to the effective oxidation of all species to As^V. Limits of detection (DLs) were calculated as three times the standard deviation of a blank injected ten times, divided by the sensitivity (slope of the calibration graph). DLs values of 0.08, 0.1, 0.1 and 0.3 $\mu g\ l^{-1}$ were obtained without the photooxidation step for As^{III}, DMA, MMA and As^V, respectively. The inclusion of the photooxidation step, resulted in DLs of 0.3 $\mu g\ l^{-1}$ for all the species considered. Repeatability was calculated after seven consecutive injections of the same standard solution, with RSD values between 3 and 6%. Reproducibility values ranging between 6 and 10% RSD were obtained after seven injections on non-consecutive days.

3.2 Solvent removal procedures

Arsenic species were extracted from biota tissues employing methanol, water or a mixture of both solvents. The methanol content had to be removed because it produced some distortion in the chromatographic separation, with the result of some peak shape distortion and a reduction in the retention times. A N_2 stream and rotatory evaporation were tested to remove the solvent before the chromatographic determination. In these experiments, 100 ng of each arsenic species was dissolved in 40 g of methanol and then the solvent was removed using either a N_2 stream at room temperature or a rotatory evaporator at 30 °C, respectively. The arsenic species were redissolved adding distilled water to a final weight of 20.0 g and analyzed for arsenic species. No significant differences were found using both concentration methods and a solution of water containing the same amount of each arsenic species (ANOVA, $P > 0.8$). Rotatory evaporation was used in further experiments as a faster concentration was achieved.

3.3 Clean-up procedures

It is a common procedure to eliminate the organic matter content present in biota samples prior to the chromatographic separation, due to possible interferences and the loss of resolution between the chromatographic peaks. Different clean-up procedures have been reported by several authors, although in most cases no data were reported to support their selection.[8,20,24] Moreover, no comparison has been performed between them. In order to evaluate the influence of the clean-up procedures, two sets of experiments were conducted with different amounts of homogenized biota tissues (1.00 g of cockles, 1.00 g of mussels, 5.00 g of oysters and 5.00 g of algae), extracted using: (i) sonication during 20 min with 20 ml of methanol and (ii) sonication with 20 ml of (1 : 1) methanol–water mixtures. Each sample was extracted twice, the centrifuged supernatants decanted, the methanol removed and the two extractants gathered. Distilled water was added to a final weight

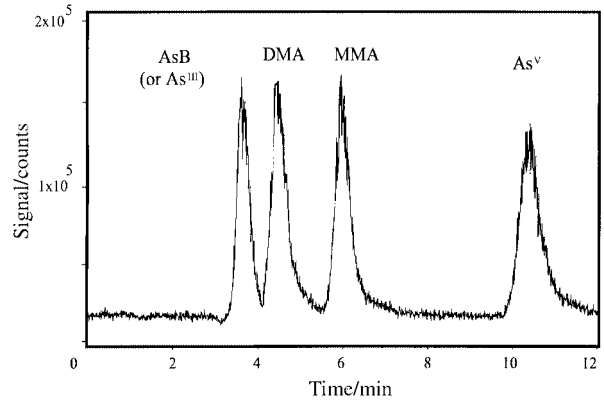

Fig. 2 HPLC-(UV)-HG-ICP-MS traces for arsenic species, each at 10 $\mu g\ l^{-1}$ (as As).

of 20.0 g. In order to evaluate the influence of the biological matrix on the clean-up procedure, the resulting solutions were spiked with 5 μg l^{-1} of each arsenic species and several clean-up procedures were tried: filtration with a 0.45 μm membrane filter, ultrafiltration with 10 000 MWCO filters at 3000*g*, 600 mg C-18 cartridges (conditioned with methanol) and 10 ml hexane partitioning. The results showed no differences in the recoveries for all the arsenic species for oyster and alga samples using either methanol or methanol–water mixtures and the four clean-up procedures (ANOVA, $P > 0.6$). Moreover, no differences were found for mussel and cockle samples, when the methanol–water (1 : 1) mixture and the four clean-up procedures were compared (ANOVA, $P > 0.09$). Similar results were obtained by Albertí *et al.*[22] for one of the clean-up procedures (C-18 cartridges), who did not observe arsenic losses with methanol–water mixtures (1 : 1) for mussel samples.

On the other hand, up to 25% lower results were obtained in our study for mussels and cockles when methanol instead of the (1 : 1) methanol–water mixture was used in the previous extraction step. In this case, of the different clean-up procedures tried, the best results for mussels and cockles were obtained using hexane partitioning, as can be seen in Table 1. Therefore, hexane partitioning was selected as the clean-up procedure for further experiments.

3.4 Sample extraction

3.4.1 Sonication. On the basis of the previous results, portions of 1.00 g of mussel and 5.00 g of alga tissues were sonicated during 20 min using 20 ml of different ratios of methanol–water mixtures: (1 : 3), (1 : 1), (3 : 1) and pure methanol. Mussel samples were spiked with MMA to a final concentration of 5 μg kg^{-1}, due to its absence in both samples. In all cases, methanol was removed by rotatory evaporation, made up to a final weight of 20.0 g with water and cleaned-up with hexane. As can be seen in Fig. 3, a maximum recovery was achieved above 50% of methanol (*t*-test, $P < 0.01$) for the alga and above 75% of methanol for the mussel ($P < 0.04$). In order to assure the maximum arsenic species extraction, 100% methanol is recommended in the sonication step. This result did not contradict previous work with sonication,[20] that proposed the use of sonication with a (3 : 1) mixture of methanol–water for arsenic extraction from biological freeze-dried samples. In our case, wet samples were used, so if their water content is considered, the proportion of methanol–water is close to the one proposed.

Extraction times between 15 and 40 min were tried, using 20 ml of methanol as extractant, with an optimum value of 20 min. The number of repetitive extractions until no arsenic was extracted was also investigated. For mussels, oysters and cockles, two extractions were needed, in accordance with the results from other authors[20,22] who tried 2–5 extractions. The extraction of the fresh water algae was more cumbersome, four

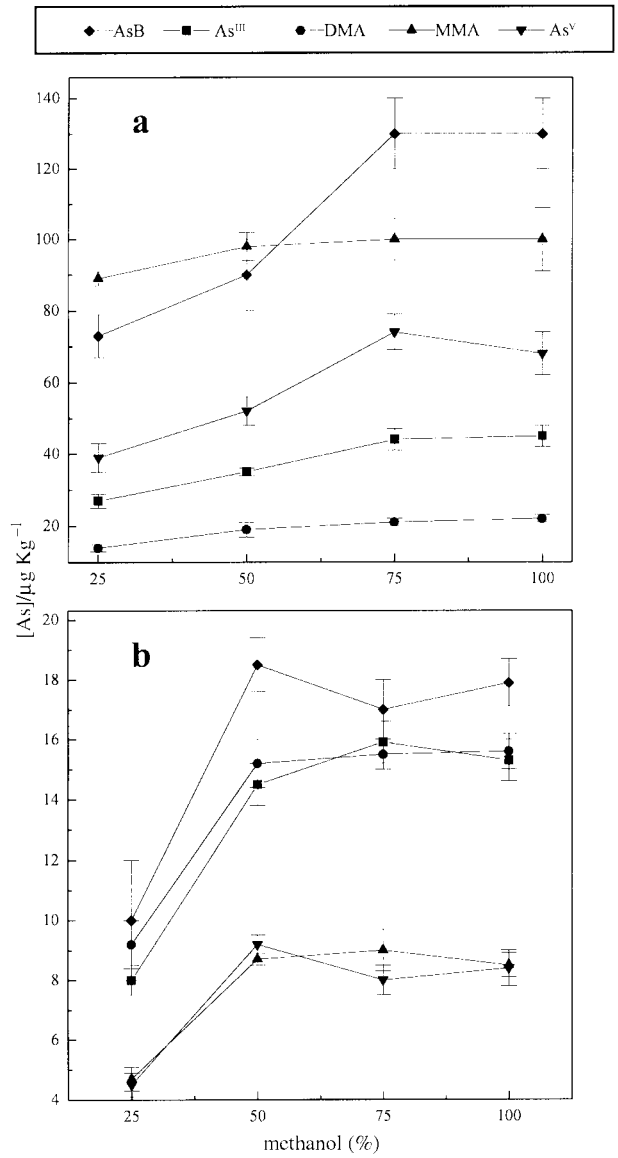

Fig. 3 One single extraction using sonication and different proportions of methanol–water mixtures: (a) alga sample; (b) mussel sample.

Table 1 Arsenic species concentrations (as μg As g^{-1} solution) ± standard deviation using different clean-up procedures for mussel and cockle samples. Extraction performed using sonication with 20 ml of methanol, rotatory evaporation and redissolution with water to a final weight of 20 g

Sample	Species	Filtration 0.45 μm	Ultrafiltration 10 000 MWCO	C-18 cartridges	Hexane partitioning
Mussel					
	AsB	134 ± 9	138 ± 9	138 ± 9	160 ± 20
	As^{III}	44 ± 3	45 ± 3	47 ± 2	59 ± 4
	DMA	24 ± 1	22 ± 1	24 ± 1	29 ± 1
	MMA	77 ± 4	77 ± 7	80 ± 3	99 ± 3
	As^{V}	82 ± 3	82 ± 5	81 ± 5	93 ± 3
	Unknown	1310 ± 9	1400 ± 60	1500 ± 80	1820 ± 20
Cockle					
	AsB	107 ± 4	108 ± 3	104 ± 4	106 ± 7
	As^{III}	30 ± 2	30 ± 1	31 ± 2	32 ± 2
	DMA	81 ± 5	83 ± 4	83 ± 5	97 ± 8
	MMA	81 ± 5	78 ± 4	80 ± 5	107 ± 4
	As^{V}	110 ± 5	109 ± 7	114 ± 7	104 ± 4
	Unknown	960 ± 50	910 ± 50	930 ± 50	1000 ± 100

repetitive extractions being necessary for quantitative recovery. Results corresponding to mussels and cockles are depicted in Fig. 4.

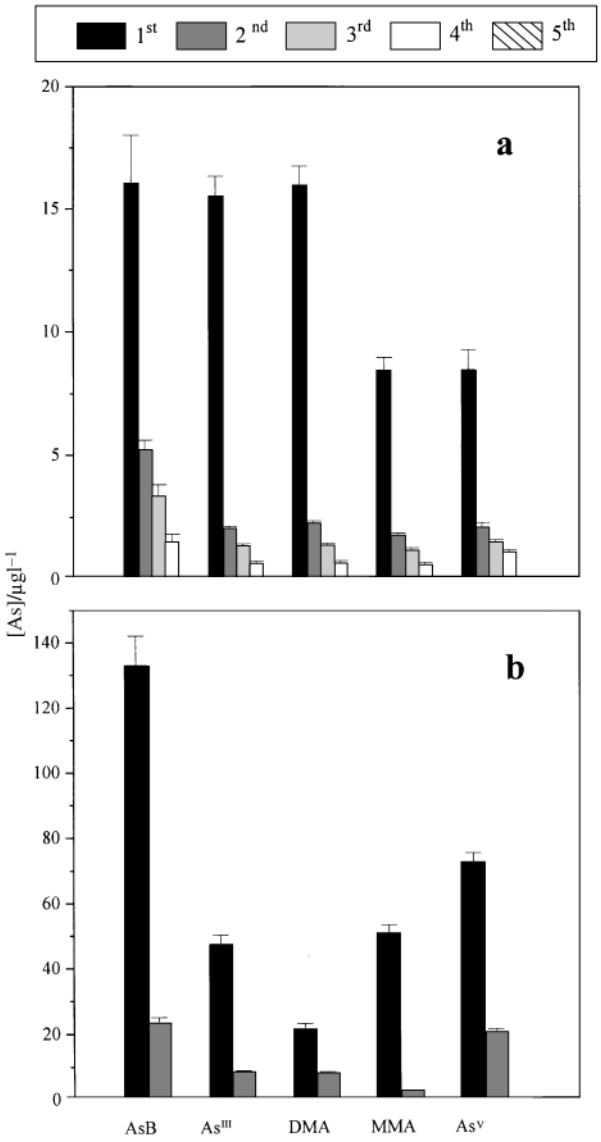

Fig. 4 Repetitive extractions using sonication with 100% methanol: (a) alga sample; (b) mussel sample.

3.4.2 Microwave. Portions of 1.00 g of mussel and 5.00 g of alga samples were extracted for a total time of 10 min in a domestic microwave at intervals of 1 min at 150 W (with 30 min of cooling to room temperature between intervals), using methanol and methanol–water (1 : 1) mixtures as extractants. Recoveries for the mussel sample after three repetitive extractions (each of 10 min) were 17–33% lower for all species than those obtained using sonication, whereas no significant differences between microwave extraction and sonication were observed for the alga sample (*t*-test, $P > 0.07$). Also, no significant differences were found for both type of samples with microwave radiation using either methanol or the methanol–water (1 : 1) mixture (*t*-test, $P > 0.08$). Unluckily, the cooling time between each 1 min extraction made this procedure tedious.

3.4.3 Soxhlet. Portions of 1.00 g of mussel and 5.00 g of alga samples were treated by Soxhlet extraction during 16 h, using 150 ml of either methanol or methanol–water (1 : 1) mixture. The solvent was removed by rotatory evaporation, and the residue made up to a final weight of 10 g with water. For both samples, better extraction yields for all the species were obtained with the methanol–water mixture compared to the methanol (*t*-test, $P < 0.02$), the results being remarkably higher for the alga sample, as can be seen in Table 2.

4 Sample analysis

The content of arsenic species in the biota samples was evaluated selecting two of the most favourable extraction methods considered in this work: (i) Soxhlet extraction with 150 ml (1 : 1) of methanol–water during 16 h and (ii) repetitive extractions using sonication with 20 ml of methanol during 20 min. Results, expressed as μg As kg^{-1} (dry weight) of sample are summarized in Table 3.

It can be noticed that the results for each extraction method depended on the sample considered. This is quite notorious for the alga sample, which gave very low recoveries (22%) using sonication compared to Soxhlet extraction (96%). Cockle samples showed a similar behaviour, but not so marked (76% *vs.* 91%). No significant differences in the extraction percentages were observed for the oyster and mussel samples, with recoveries in all cases over 90%. These recoveries were higher than those reported by other authors, ranging between 85% and 60% for bivalve samples.[22,10] In general, our study provided

Table 2 Arsenic species concentrations (as μg As g^{-1} solution) ± standard deviation obtained using different extraction procedures based on 20 ml of either methanol or (1 : 1) methanol–water mixtures, concentration with rotatory evaporation and dilution with water to a final weight of 20 g

Procedure	Sample	AsB	As^{III}	DMA	MMA	As^{V}	Unknown
Sonication	Mussel	156 ± 11[a]	55 ± 3	29 ± 2	nd[e]	92 ± 4	1720 ± 110
		168 ± 9[b]	52 ± 3	27 ± 3	nd	93 ± 3	1810 ± 90
	Alga	26 ± 3[c]	19 ± 1	20 ± 1	12 ± 1	13 ± 1	nd
		28 ± 3[d]	18 ± 2	22 ± 1	13 ± 2	15 ± 2	nd
Microwave	Mussel	96 ± 8[a]	40 ± 4	24 ± 2	38 ± 3	61 ± 4	1045 ± 105
		106 ± 9[b]	47 ± 4	22 ± 1	43 ± 2	62 ± 4	1117 ± 72
	Alga	28 ± 1[c]	19 ± 1	24 ± 1	13 ± 1	16 ± 1	nd
		31 ± 3[d]	18 ± 1	24 ± 1	13 ± 1	17 ± 1	nd
Soxhlet	Mussel	93 ± 6[a]	43 ± 2	24 ± 1	nd	57 ± 3	1380 ± 70
		180 ± 1[b]	55 ± 3	31 ± 2	nd	95 ± 7	2000 ± 100
	Alga	30 ± 2[c]	19 ± 1	22 ± 1	13 ± 1	14 ± 1	nd
		93 ± 6[d]	97 ± 5	46 ± 2	20 ± 1	79 ± 5	nd

[a] 1 g of mussel extracted with methanol. [b] 1 g of mussel extracted with a methanol–water (1 : 1) mixture. [c] 5 g of alga extracted with methanol. [d] 5 g of alga extracted with a methanol–water (1 : 1) mixture. [e] Not detected

Table 3 Arsenic species concentrations (as μg As g^{-1}, dry weight basis) ± standard deviation in biota samples

Sample	As^{III}	DMA	MMA	As^{V}	AsB	Unknown	$As_{extracted}$	As_{total}[f]	Percentage extracted
Alga	8 ± 1[a]	20 ± 1	5 ± 0.3	11 ± 1	23 ± 3	nd	77 ± 3	350 ± 10	22
	97 ± 5[b]	46 ± 2	20 ± 1	79 ± 5	93 ± 6	nd	335 ± 10	350 ± 10	96
Mussel	54 ± 4[a]	31 ± 2	nd	95 ± 6	160 ± 20	2200 ± 200	2540 ± 201	2700 ± 100	94
	55 ± 3[b]	31 ± 2	nd	95 ± 7	180 ± 10	2200 ± 200	2561 ± 200	2700 ± 100	95
Cockle	78 ± 4[c]	nd[d]	nd	240 ± 10	180 ± 20	1620 ± 70	2118 ± 74	2800 ± 200	76
	114 ± 6[b]	nd	nd	340 ± 30	200 ± 20	1900 ± 100	2554 ± 106	2800 ± 200	91
Oyster	22 ± 1[c]	39 ± 4	nd	32 ± 2	180 ± 10	2800 ± 100	3073 ± 101	3200 ± 100	96
	22 ± 2[b]	37 ± 2	nd	31 ± 1	180 ± 10	2700 ± 200	2970 ± 200	3200 ± 100	92
CRM 627627	nd	1.8 ± 0.2[c,e]	nd	nd	50 ± 2[e]	nd	51.8 ± 2[e]	54.0 ± 3[e]	94
	nd	1.9 ± 0.2[b,e]	nd	nd	51 ± 1[e]	nd	52.9 ± 1[e]	54.0 ± 3[e]	96
TORT-1-1	nd	1700 ± 100[c]	nd	860 ± 60	17 000 ± 1000	nd	19 560 ± 1006	24 600 ± 1600	80
	nd	1600 ± 100[b]	nd	770 ± 60	15 000 ± 900	nd	17 370 ± 907	24 600 ± 1600	71

[a] Four repetitive extractions, using sonication with 20 ml of methanol. [b] Soxhlet extraction with 150 ml of methanol–water (1 : 1) during 16 h. [c] Two repetitive extractions, using sonication with 20 ml of methanol. [d] Not detected. [e] Results expressed as μmol k^{-1}. [f] After digestion with 10 ml of HNO_3–HCl (3 : 1) and 5 ml of $HClO_4$.

better results for all the samples using the procedure based on Soxhlet extraction, with the only exception of the reference material TORT-1, which gave higher percentages using sonication (80%) instead of Soxhlet extraction (71%). The results obtained for the CRM 627 using both procedures were quite similar, with extraction yields of 94% and 96% with respect to the certified values for DMA and AsB, respectively.

Inorganic arsenic represented a minor fraction (3–16%) of the total arsenic in the marine samples, arsenate being the predominant species. However, the opposite was found for the riverine alga sample, where inorganic arsenic represented up to 50% of the total arsenic, arsenite being the most abundant species.

Of the two methylated species considered in this work, MMA was only found in the alga. On the other hand, quite similar concentrations of DMA were found in mussel, alga and oyster, being not detected in the cockle. DMA also represented the second most abundant species in TORT-1 and CRM 627. In this latter material, the DMA concentration found with both extraction procedures (1.8 ± 0.2 and 1.9 ± 0.2 μmol kg^{-1}) did not statistically differ from the certified value (2.0 ± 0.3 μmol kg^{-1}).

All the studied samples contained arsenobetaine in some degree. It is the main arsenic compound present in TORT-1, as has been described by Larsen *et al.*[28] In our study, the concentration of AsB found in TORT-1 (17 ± 1 μg g^{-1} as As) does not differ significantly from the value reported by Larsen *et al*. The AsB concentrations obtained for CRM 627 (50 ± 2 μmol kg^{-1} and 51 ± 1 μmol kg^{-1} were in good agreement with the certified value 52 ± 3 μmol kg^{-1}). Also, the three marine bivalves contained about the same quantity of arsenobetaine (180–200 μg kg^{-1} as As), and the fresh water alga about half of that quantity. The presence of arsenobetaine in the fresh water alga is noticeable, because previous workers have reported the presence of one arsenosugar in a type of terrestrial alga, but not of arsenobetaine.[29]

It is also worth mentioning the presence of an unknown arsenic peak (Fig. 5b) in the three bivalve samples, which was present at higher concentrations by far than any of the inorganic (As^{III}, As^{V}) or organic (DMA, MMA and AsB) species considered in this study. This can be attributed to the presence of either trimethylarsinic oxide[20] (as a result of AsB photolysis) or some arsenosugars in this type of sample, as has been pointed out by some authors.[8,10,21] Unfortunately, the identification of this type of compound presents the handicap of the lack of commercially available standards. However, some authors[23] have described the presence of some arsenosugars in reference materials, namely NIST 1566a (oyster tissue). Unluckily, this could not be tried in the present work, due to the fact that this material is not commercially available at the moment.

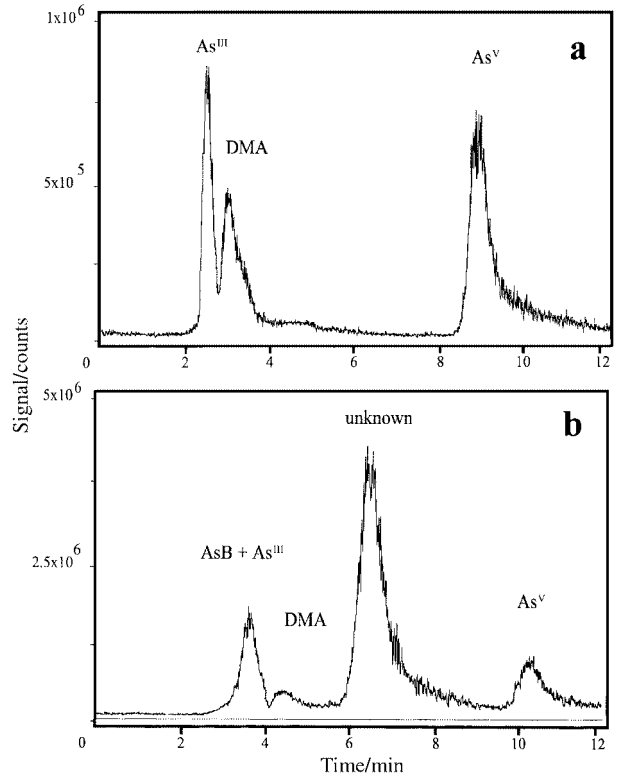

Fig. 5 Soxhlet extraction of mussel sample, using: (a) HPLC-HG-ICP-MS; (b) HPLC-(UV)-HG-ICP-MS.

5 Conclusions

The extraction and the clean-up procedures are critical steps for high species recoveries in arsenic speciation, depending on the type of sample considered (mussel, cockle, oyster and alga). Of the different extraction procedures tried in this work for the analysis of biota samples, Soxhlet extraction with 150 ml of (1 : 1) methanol–water mixture and sonication with 20 ml of methanol provided the best arsenic recoveries. The results with these two extraction procedures showed the presence of an unidentified arsenic species which represented the main form of this element in the marine samples considered in this work (oyster, cockle and mussel). Arsenobetaine was also present at high concentration in these samples. Inorganic arsenic was found in the three marine samples, whereas MMA was not identified in any of them. The cockle sample did not contain DMA either. On the other hand, the fresh water alga presented comparable levels of both inorganic arsenic (arsenite and

arsenate) and arsenobetaine. Also, it was the only sample that contained both methylated arsenic forms (DMA and MMA).

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Paper a908884i